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Antibody responses to liposome-associated antigen

W. E. Vannier[1]* and S. L. Snyder[2]

[1]*Naval Medical Research Institute, Bethesda, MD 20854, U.S.A. and* [2]*Naval Research Laboratory, Code 6100.2, Washington, DC 20375-5000, U.S.A.*

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1. Summary

The humoral antibody response of CAF1 mice to low doses (1–100 μg) of egg albumin (EA) encapsulated in or covalently bound to the surface of liposomes was studied for three routes of administration. The liposome immunoadjuvant effect observed was found to depend on the location of the antigen, either on the liposome surface or entrapped inside the liposome, and on the number of immunizations. Following a single immunization, the highest antibody titers were elicited with liposomes having EA conjugated to their surface, regardless of the route of administration. For multiple immunizations given i.v. or i.p. EA conjugated to the surface of liposomes was also superior to either free or liposome-encapsulated EA. However, the antibody response to EA bound to the surface of liposomes was not enhanced as compared to free EA following multiple subcutaneous immunizations.

Key words: Liposomes; Immunopotentiation; Egg Albumin; Surface antigen; Adjuvant

Abbreviations: ELISA, enzyme-linked immunosorbent assay; EA, egg albumin; s.c., subcutaneous, i.v., intravenous; i.p., intraperitoneal; RES, reticuloendothelial system

Correspondence to: S. L. Snyder, Naval Research Laboratory, Code 6100.2, Washington, DC 20375-5000, U.S.A.

* *Current address:* Division of Chemistry and Chemical Engineering, California Institute of Technology Pasadena, California 91125, U.S.A.

2. Introduction

We have previously reported a simple method for covalently attaching antigen (egg albumin) to the surface of preformed multilamellar liposomes by diazotization of surface arylamines and coupling to the protein antigen [1]. Data were also presented showing that antigen covalently bound to the surface of the liposomes was more effective than liposome-encapsulated antigen or free antigen in inducing an antibody response after a single intravenous immunization. These results supported the idea that surface presentation of antigen played an important role in the immunoadjuvant effect of liposomes [1–3]. On the other hand, evidence has been presented suggesting that association of the antigen with the surface of liposomes may not be required for immunopotentiation [4–6]. Due to the wide range of experimental conditions used in the aforementioned investigations, it is difficult to draw firm conclusions as to the relative immunogenicity of surface-bound antigen vs. that which is entrapped within liposomes. Consequently, we have extended our previous study by comparing antibody responses to free egg albumin (EA), liposome-encapsulated EA and EA covalently bound to the surface of preformed liposomes, as a function of several parameters. In particular, our results show that the humoral response of mice to antigen which is encapsulated in or bound to the surface of liposomes is highly dependent on the route of administration as wel as the type of response observed (primary vs. secondary).

3. Materials and Methods

The cholesterol and egg yolk *L*-α-

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phosphatidylcholine used to prepare liposomes, and the *p*-nitrophenyl stearate and *p*-phenylenediamine used to make *N*-(*p*-aminophenyl)stearylamide for attaching egg albumin to the liposome surface, were obtained from Sigma Chemical Co., (St. Louis, MO). Egg albumin, 5× crystallized (EA), and bovine serum albumin fraction V were obtained from Calbiochem (La Jolla, CA). Sephadex G-200 and Sepharose 4B were obtained from Pharmacia Fine Chemicals Inc., (Piscataway, NJ).

3.1. *Preparation of the liposomes*

N-(*p*-aminophenyl)stearylamide was synthesized as previously described [1]. Multilamellar liposomes were prepared with egg yolk *L*-α-phosphatidylcholine (15 μmol), cholesterol (7.5 μmol), and *N*-(*p*-aminophenyl)stearylamide (1.1 μmol). A film of the lipids dried in a 100-ml round-bottom flask was resuspended in 10 ml of borate saline (0.17 M sodium chloride/0.01 M borate buffer, pH 8.0) by shaking and the use of a Vortex mixer. After one hour at room temperature, liposomes were pelleted at 20000×*g* for 10 min, resuspended in 2.0 ml of 0.2 M sodium nitrite and diazotized by the addition of 0.2 M sodium chloride with 0.2 M hydrochloric acid at 4 °C. After 5 min the diazotized liposomes were pelleted at 20000×*g* for 5 min, and coupled with EA (2.5 mg/ml) in 0.05 M borate buffer, pH 10, at 4 °C. The mixture was placed in an ice bath and allowed to come to room temperature overnight.

The tan-colored liposomes were washed three times with 0.17 M sodium chloride/0.01 M borate buffer, pH 8.0, by centrifugation. The amount of EA used to achieve a particular level of protein binding to the liposome surface was based on model experiments using ^{125}I-labeled EA.

3.2. *Animals*

Female CAF1 mice from Jackson Laboratories, (Bar Harbor, ME) 7 to 14 weeks of age were used for the immunization experiments. Mice were immunized by intravenous (i.v.), intraperitoneal (i.p.) or subcutaneous (s.c.) injection of 0.1 ml volumes of free EA or liposome-associated EA in 0.17 M sodium chloride/0.01 M borate buffer, pH 8.0. The mice were bled from the retro-orbital plexus and the serum samples stored frozen at −20 °C until used in the ELISA.

3.3. *Enzyme-linked immunosorbent assay (ELISA)*

The serum antibody responses to EA were assayed using a microtiter plate ELISA as previously described [1]. Imulon U-bottom polystyrene plates (Cook Laboratory Products, Alexandria, VA) were coated with 40 μg EA per well in coating buffer (0.1 M sodium carbonate, 0.02% sodium azide, pH 9.6). The plates were washed 5 times with working buffer (2.2 g boric acid, 0.2 g sodium hydroxide, 9.29 g sodium chloride, 0.09 g sodium azide, 5.0 g Tween-20 and 5.0 g boven serum albumin fraction V per liter of solution with pH adjusted to 7.8 with hydrochloric acid). To reduce nonspecific binding, 2.0% bovine serum albumin in working buffer was added to the wells for 30 min.

After washing five times with working buffer, serum dilutions in working buffer were added in 0.1-ml volumes. After 2 h in a humidified chamber at room temperature, the plates were washed five times with working buffer and 0.1 ml volumes of a suitable dilution (generally 1/1000) of an alkaline phosphatase-labeled immunoadsorbent-purified goat anti-mouse IgG (H + L) (Sigma Chemical Co.) were added. Following incubation at room temperature for 2 h in a humidified chamber, the plates were washed 5 times with working buffer and once with diethanolamine buffer (97 ml diethanolamine buffer: 97 ml diethanolamine, 0.2 g sodium azide, 0.1 g $MgCl_2 \cdot 6H_2O$ and 800 ml water). Volumes of 0.1 ml of substrate solution (1 mg/ml of *p*-nitrophenylphosphate, Sigma Chemical Co., dissolved in the diethanolamine buffer) were added to each well and the plates incubated 1 h at room temperature. To stop the reaction, 0.15 ml volumes of 2 M sodium hydroxide were added to each well and the absorbance read at 406 nm with an ELISA plate reader (Dynatech Model MR 580, Dynatech Laboratories, Inc., Alexandria, VA).

In each assay a standard serum pool of mouse anti-EA antibody was run at three concentrations (1:10000, 1:20000 and 1:40000). Each serum sample was run in duplicate in two assays. In order to estimate the antibody concentration of the standard pool, immunoadsorbent purified mouse anti-EA

antibody was prepared by the use of a Sepharose 4B-EA derivative prepared by cyanogen bromide activation and coupling [7]. The anti-EA antibodies were allowed to bind to the adsorbent, the non-specific serum proteins removed by washing with the borate saline pH 8.0 and the anti-EA antibody eluted with glycine-HCl buffer pH 2.35 [8]. The eluted antibody was immediately adjusted to pH 7.8, concentrated in a dialysis sac with dry Sephadex G 200 and dialyzed against borate saline, pH 8.0.

The protein concentration of the eluted antibody solution was determined by measuring the absorbance at 280 nm ($E^{1\%} = 14$). ELISA experiments were carried out with the specifically purified antibody and the standard pool. The antibody content of the standard pool was estimated by comparison of the ELISA absorbance and assuming the purified antibody was 100% anti-EA antibody. Previous studies using a similar method with the bovine serum albumin-rabbit antibody system have indicated that 95% or more of the protein eluted was antibody [8]. The standard serum was found to contain about 600 μg of antibody per ml. Experimental serum antibody concentrations were reported based on a comparison of ELISA absorbance values with the standard antibody pool for each microtiter plate. Duplicate antibody concentrations from the two assays for each serum were averaged. The assay values for normal serum showed some variation but were generally less than 0.3 μg. Most of the variation in the antibody assays for the groups of mice reflects differences in the immune responses of the individual mice within the group.

4. Results

4.1. *Immunization with a single dose of free or liposome associated antigen*

The temporal response of serum antibody titers to a single injection of free EA or EA that was either encapsulated in or covalently bound to the surface of liposomes was studied for three routes of administration (i.v., i.p. and s.c.). The results of these studies are summarized in Table 1. The antibody levels following a single intravenous immunization using three different methods of antigen presentation were consistent with the responses we reported previously using slightly higher doses of EA [1].

Surprisingly, the EA that was 'encapsulated' in liposomes was not found to be significantly more antigenic than 'free' EA. In fact, it appeared that s.c. administration of liposome-encapsulated EA may have actually diminished the antibody response relative to the free antigen. In contrast, EA covalently attached to the surface of liposomes elicits responses that were both earlier and of a greater magnitude than non-liposome associated EA (Table 1). The immunoadjuvant effect resulting from the conjugation of EA to the surface of liposomes was observed for all three routes of administration. In further experiments, mice were immunized i.v. or s.c. with single doses of EA or liposome-diazo-EA over a range of 1 to 100 μg EA, and antibody concentrations determined two weeks after immunization (Table 2). Comparison of the antibody titers in these experiments showed that covalent attachment of EA to the surface of liposomes elicited elevated antibody levels at doses significantly less than those needed to produce similar responses using free EA.

4.2. *Immunization with multiple doses of free EA or liposome-associated EA*

Mice were immunized three times by different routes with three 1 μg doses of free EA, EA encapsulated in liposomes or covalently bound to the surface of preformed liposomes. Half the mice were immunized at one-week intervals and the other half at intervals of four weeks. All the mice were bled two weeks after the last immunization. The data are presented in Table 3. A comparison of the three routes of immunization indicates that higher antibody responses were obtained by multiple s.c. immunizations with EA or liposome-diazo-EA.

It was especially striking that liposome encapsulated antigen generally gave lower responses than either free antigen or liposome surface-bound antigen except when administered i.v., where it was more effective than the free antigen. As shown in Table 1, a single subcutaneous immunization with the liposome-bound antigen provided more effective immunization than a single dose of free antigen. Immunization with multiple subcutaneous doses of free EA was somewhat more effective than with the liposome bound antigen. In the case of liposome-diazo-EA, the four-week immunization interval was more effective than the one-week interval (Table 3).

TABLE 1

Serum antibody concentrations after a single intravenous, intraperitoneal or subcutaneous immunization of CAF1 mice with free EA or liposome-associated EA.

Group description	Dose (μg)	Mean antibody concentrations ± SEM, μg/ml			
		1 week	2 weeks	4 weeks	6 weeks
i.v. immunization					
Free EA	5.0	0.5 ± 0.04	0.3 ± 0.06	0.5 ± 0.05	0.3 ± 0.03
	1.0	0.7 ± 0.3	0.3 ± 0.02	0.5 ± 0.1	0.2 ± 0.03
Liposome-encapsulated EA	5.0	1.2 ± 0.8	0.4 ± 0.02	0.4 ± 0.06	0.2 ± 0.02
	1.0	0.4 ± 0.07	0.9 ± 0.4	0.5 ± 0.02	0.3 ± 0.02
Liposome-diazo-EA	5.0	2.4 ± 0.17	9.4 ± 2.7	15.9 ± 0.9	18.7 ± 3.7
	1.0	0.7 ± 0.1	3.6 ± 0.4	14.5 ± 3.1	4.6 ± 0.8
i.p. immunization					
Free EA	5.0	0.4 ± 0.02	1.6 ± 1.1	0.4 ± 0.06	0.4 ± 0.08
	1.0	0.4 ± 0.08	0.4 ± 0.03	0.4 ± 0.07	0.3 ± 0.06
Liposome-encapsulated EA	5.0	0.4 ± 0.07	0.4 ± 0.06	0.3 ± 0.01	0.6 ± 0.2
	1.0	0.5 ± 0.2	0.5 ± 0.2	0.4 ± 0.04	0.3 ± 0.06
Liposome-diazo-EA	5.0	0.5 ± 0.08	0.8 ± 0.3	7.2 ± 2.0	9.5 ± 4.0
	1.0	0.5 ± 0.08	0.8 ± 0.1	3.7 ± 1.5	5.0 ± 2.0
s.c. immunization					
Free EA	5.0	0.4 ± 0.03	2.7 ± 0.6	2.0 ± 0.4	2.1 ± 0.9
	1.0	0.5 ± 0.1	0.5 ± 0.06	0.7 ± 0.2	0.3 ± 0.03
Liposome-encapsulated EA	5.0	0.4 ± 0.06	0.7 ± 0.1	0.7 ± 0.2	0.3 ± 0.03
	1.0	0.5 ± 0.02	0.4 ± 0.05	0.6 ± 0.1	0.4 ± 0.06
Liposome-diazo-EA	5.0	0.6 ± 0.07	1.4 ± 0.08	15.5 ± 8.3	10.4 ± 6.0
	1.0	0.5 ± 0.09	0.4 ± 0.05	0.6 ± 0.08	0.7 ± 0.3

Mice were immunized with single 1.0 or 5.0 μg doses of free EA, EA encapsulated in liposomes or EA covalently coupled to the surface of preformed liposomes. Groups of five mice were bled at one, two, four or six weeks.

TABLE 2

Serum antibody responses with different antigen doses after a single intravenous or subcutaneous immunization of CAF1 mice with free EA or EA-diazo-linked to liposomes.

Immunization route	Dose EA, μg	Mean antibody concentrations ± SEM, μg/ml	
		Free EA	Liposome-diazo-EA
i.v.	1	–	2.6 ± 1.9
	5	0.7 ± 0.3	23.3 ± 1.6
	10	0.5 ± 0.1	27.4 ± 4.3
	20	0.5 ± 0.08	19.8 ± 5.2
	100	5.7 ± 5.0	–
s.c.	1	–	4.3 ± 2.0
	5	0.5 ± 0.03	11.6 ± 6.2
	10	1.2 ± 0.4	22.6 ± 11.8
	20	4.2 ± 0.5	38.8 ± 11.8
	100	22.8 ± 14.0	–

Groups of five mice were immunized with from 1 to 100 μg of free EA or EA coupled to the surface of preformed diazotized liposomes. All the mice were bled two weeks after immunization.

5. Discussion

In the development of vaccines, liposomes would appear to be one of the most flexible carriers for antigen presentation [9, 10], and to be of particular value as vehicles for immunization with membrane-derived antigens [11]. Diazotization and coupling reactions provide a convenient method for the attachment of many different kinds of antigens to single or multilamellar liposomes. Furthermore, using liposomes as antigen carriers provides the possibility of encapsulating water-soluble immunopotentiating agents inside liposomes and/or incorporating hydrophobic components such as Lipid A or lipophilic muramyl dipeptide in liposome membranes [12].

In our experiments, measuring the antibody response after a single immunization, we have found liposome surface-bound EA a more effective immunogen than free EA or liposome-encapsulated

TABLE 3

Serum concentrations of antibody after three intravenous, intraperitoneal or subcutaneous immunizations of CAF1 mice with free EA or liposome-associated EA.

	Mean antibody concentrations ± SEM, μg/ml	
	One-week interval	Four-week interval
i.v. immunization		
Free EA	1.0 ± 0.7	0.3 ± 0.02
Liposome-encapsulated EA	20.7 ± 7.2	6.2 ± 3.6
Liposome-diazo-EA	46.1 ± 4.9	175.0 ± 12.2
i.p. immunization		
Free EA	56.9 ± 31.6	6.6 ± 3.3
Liposome-encapsulated EA	1.2 ± 0.5	1.3 ± 0.2
Liposome-diazo-EA	27.5 ± 12.8	247.0 ± 61.7
s.c. immunization		
Free EA	204.0 ± 80.6	530.0 ± 191
Liposome-encapsulated EA	2.7 ± 1.7	1.1 ± 0.8
Liposome-diazo-EA	80.2 ± 15.9	314.0 ± 63.5

Groups of five mice were immunized three times with 1.0 μg doses of free EA, liposome-encapsulated EA or EA diazo-linked to the surface of preformed liposomes. In one part of the experiment the mice were immunized three times at one-week intervals while the remaining mice were immunized with an interval of four weeks. All the mice were bled two weeks after the last immunization.

EA, regardless of the route of immunization. No significant enhancement of the primary immune response was observed with liposome-encapsulated EA. Previous studies [10] reporting enhancement of immune responses to antigen by encapsulation in liposomes have generally employed higher doses of antigen than those routinely used in our investigations (1–10 μg). The antigen doses used in this study were low and this may be responsible for our inability to demonstrate an adjuvant effect with the liposome-entrapped antigen. However, in a recent study Latif and Bachhawat [13] compared the primary immune response of the rabbits to lysozyme (s.c.) entrapped in or covalently bound to neutral liposomes and found significant enhancement only in the latter case. These findings are in substantial agreement with the results presented in Tables 1 and 2. Whether single or multiple immunizations were performed, when the route of administration was i.v., antigen covalently bound to the surface of liposomes elicited the highest antibody titers (Tables 1 and 3).

The mechanism(s) by which liposomes promote the immune response of encapsulated or surface conjugated antigens is unknown. However, it seems reasonable to suggest that the mechanism of liposome immunopotentiation may be different for different routes of administration. In the case of a single s.c. immunization our results (Table 1) are consistent with the notion that liposomes are promoting the response to surface bound EA via a 'depot' effect. In contrast, the early elevated response observed with liposome-diazo-EA following a single i.v. immunization (Table 1) is more consistent with a mechanism involving enhanced RES macrophage uptake and processing [10, 14].

The data for the antibody responses after multiple immunizations showed high responses for the liposomal bound antigen by all three routes and for free antigen given subcutaneously (Table 3). Multiple subcutaneous immunizations with free antigen were found to give a slightly better humoral antibody response than with the same amount of antigen bound to the surface of liposomes. These results are also consistent with the idea that the adjuvant effect of liposomes given s.c. is due, in part, to slow release of some form of the antigen. To our surprise, encapsulating EA within liposomes markedly diminished

the secondary response to s.c. or i.p. immunizations relative to free EA. However, when the route of administration was either i.v. or i.p., coupling the antigen to the surface of liposomes clearly enhanced the secondary immune response.

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